Novel insights into the effect of paroxetine administration in pilocarpine-induced chronic epileptic rats

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Abstract. The aim of the present study was to investigate the role of paroxetine intervention in epilepsy, and its association with the expression of serotonin transporter (SERT) and hippocampal apoptosis. Thirty adult male Sprague Dawley rats were divided into control vehicle (n=6) and epileptic (n=24) groups. Status epilepticus (SE) was induced via systemic injection of pilocarpine, and seizure activity was monitored via video electroencephalogram. The epileptic group was then randomly divided into two groups; Four weeks following SE induction, paroxetine (5 mg/kg/day; SE + paroxetine group) or normal saline (SE group) was intraperitoneally injected for 4 weeks. Brain tissue was collected to evaluate apoptosis via terminal deoxynucleotidyl transferase dUTP nick-end labeling. SERT, B-cell lymphoma-2 (Bcl-2) and brain derived neurotropic factor (BDNF) expression levels were evaluated by western blotting, and miR-16 expression was evaluated by reverse transcription-quantitative polymerase chain reaction. Paroxetine did not affect the mortality of the pilocarpine-induced chronic epileptic rats. Spontaneous recurrent seizures (SSRs) were observed 7-28 days following SE induction. The frequency and stage of the SSRs were reduced by paroxetine administration. Apoptotic cells were observed in the epileptic hippocampus. Following paroxetine intervention, the staining intensity and number of apoptotic cells were significantly decreased. Expression levels of BDNF and Bcl-2 were lower in the SE and number of apoptotic cells were significantly decreased. Following paroxetine intervention, the staining intensity of the SSRs were reduced by paroxetine administration. In conclusion, the seizures and hippocampal apoptosis observed in chronic epileptic rats were alleviated by paroxetine treatment. This effect may be associated with the reduced Bcl-2 and BDNF expression and the modulation of SERT expression. The alterations in miR-16 expression may provide a potential explanation for the modulation of apoptosis; however, further research is required to determine the complete underlying molecular mechanism.

Introduction

Selective serotonin reuptake inhibitors (SSRIs) were previously considered to increase the occurrence of seizures (1). Previously, clinical and experimental results indicated that SSRIs alleviate the susceptibility to seizures (2-5). This effect is attributed to elevated levels of extracellular serotonin. However, the underlying molecular mechanism of this increase remains unclear. Our previous study demonstrated a downregulation of hippocampal extracellular serotonin levels in epileptic rats and impaired serotoninergic neuronal function in raphe nucleus (6). The membrane bound serotonin transporter (SERT) serves an important role in modulating the metabolism of 5-hydroxytryptamine (5-HT). SSRIs target SERT in the raphe nucleus, decreasing serotonin reuptake and increasing the synaptic availability of serotonin. Therefore, the present study hypothesized that abnormal SERT expression may be present in epileptic models.

Paroxetine has been demonstrated to regulate the expression of B-cell lymphoma-2 (Bcl-2) and brain derived neurotropic factor (BDNF), which are associated with cell apoptosis and proliferation (7). In addition, mesial temporal lobe epilepsy (MTLE), the most common form of refractory epilepsy, is characterized by hippocampal sclerosis, including cell apoptosis and glial proliferation. Therefore, the present

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study hypothesized that paroxetine alleviates seizures by regulating both Bcl-2/BDNF and SERT.

The mechanism in which paroxetine may regulate SERT, Bcl-2 and BDNF in epilepsy remains to be fully elucidated. In recent years, an increasing number of studies, including clinical and animal experiments, demonstrated that microRNAs (miRNAs) serve an important role in the pathophysiology of epilepsy (8-11). Epileptic models are generally accompanied by selective alterations in miRNAs that regulate neuronal death, ion channels and inflammation (12-15). In a genome wide miRNA profiling study, microRNA (miR)-16 was increased in hippocampal tissue collected from patients with MTLE (15). One study revealed that paroxetine upregulates miR-16 expression in the raphe nucleus (7), and another demonstrated that Bcl-2 expression was negatively associated with miR-16 expression (16). These results were obtained using animal models of depression or tumor cells.

The present study focused on pilocarpine-induced chronic epileptic rats. Firstly, the effects of paroxetine on spontaneous recurrent seizures (SSRs) and hippocampal apoptosis was investigated. Secondly, SERT, Bcl-2 and BDNF expression levels were evaluated using western blotting, and miR-16 expression was evaluated using reverse transcription-quantitative polymerase chain reaction. Finally, the underlying molecular mechanism of miR-16 in the pathogenesis of epilepsy was investigated.

Materials and methods

Pilocarpine model of chronic epilepsy. The present study was performed in accordance with the Guide for the Care and Use of Animal Experimentation of Fujian Medical University and the Fujian Medical University Animal Ethics Committee (Fuzhou, China) specifically approved this study. Surgery was performed using 10% chloral hydrate anesthesia and efforts were made to minimize suffering. Male adult (8-10 weeks) Sprague Dawley rats (220-250 g) were housed under standard conditions (temperature, 22-26˚C; 12-h light/dark cycle; humidity, 45-50%) and had free access to food and water. Thirty rats were divided into two groups (6 rats in the vehicle group and 24 rats in the epileptic group). One week prior to the induction of status epilepticus (SE), surface electrodes were implanted into the skulls of the rats under 10% chloral hydrate anesthesia as previously described (6). A frontal electrode was implanted above the frontal cortex [coordinates, 2.5 mm frontal; 2.0 mm left and 0.5 mm deep from the bregma (17)], a second electrode was fixed to the surface of the skull as a ground electrode and a third electrode was fixed behind the ear as a reference electrode. Following the implantation procedure, animals were intraperitoneally (i.p.) injected with gentamicin to prevent infection and were allowed to recover from surgery for 1 week prior to experimentation. Twenty minutes prior to injection of pilocarpine, the muscarinic antagonist, atropine, was administered i.p. (1 mg/kg) to reduce the adverse peripheral effects of pilocarpine. The rats were injected i.p. with pilocarpine (30 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) 16-18 h after the administration of lithium (127 mg/kg, i.p.). After the drugs were administered, the progressive evolution of seizure behavior was observed and rated according to the Racine scale (18). The Racine scale was used to rate the stage of epilepsy: Stage 1 was characterized by behavioral arrest; stage 2 by head nodding, gnawing, and mild tremors; stage 3 by unilateral forelimb clonus; stage 4 by bilateral forelimb clonus; and stage 5 by severe seizures with prolonged loss of postural control or prolonged clonus. Only animals that developed stage IV and V seizures were used. SE was defined as the persistence of stage IV and V seizures for longer than 30 min (18). Electroencephalogram (EEG) potentials and the behavior of the animals were monitored using a video monitoring system (Biopac Systems Inc., Goleta, CA, USA) three times a day for 2 h each session for 8 weeks after the establishment of SE. During the chronic period, the SSRs were evaluated based on frequency (times/week) and stage (19). EEG discharges with amplitudes exceeding 50 µV, which was typically twice the basal EEG discharge amplitude, and spikes (≥70 msec) and sharp waves (70-200 msec) were counted as seizure discharges.

Intervention. A total of 18 rats survived SE induction however, four weeks after the induction of SE only 14 rats had survived. These 14 epileptic rats were divided into two sub-groups: SE and SE + paroxetine. The former group received normal saline (NS) as a control, and the latter received paroxetine. Paroxetine (5 mg/kg/day) or NS was injected i.p. for 4 weeks; only 12 rats survived during the final experiment.

Brain region isolation and morphological examinations. At the end of the experiments, the rats were deeply anesthetized (10% chloral hydrate, 2 ml/kg) and transcardially perfused with 0.1 mmol/l PBS (pH 7.4). If the tissue required fixation, the rats were perfused with PBS followed by 4% paraformaldehyde. One portion of the hippocampal tissue was used for terminal deoxynucleotidyl transferase dUTP nick-end labelling TUNEL/horseradish peroxidase (HRP) staining to visualize apoptotic cells. Another portion of the hippocampal tissue and a sample of the raphe nucleus tissue were used to evaluate expressions of SERT, Bcl-2 and BDNF via western blotting. A second set of hippocampal/raphe nucleus tissue samples was used to analyze expression of miR-16 via reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Immunohistochemical staining. The unilateral hippocampal tissue from the six rats in each of the control, SE and SE + paroxetine groups was rapidly isolated, fixed with 4% paraformaldehyde for 48 h at 4°C and embedded in paraffin. Next, the paraffin-embedded tissue was cut into sections including the cornu ammonis (CA) 1-3 and the dentate gyrus (DG) regions of the hippocampus. Subsequently, the slices were dewaxed in a series of alcohols and incubated with proteinase K (20 µg/ml) for 10 min at room temperature (22-28˚C), terminal deoxynucleotidyl transferase (50 µl) at 37°C for 60 min and an anti-biotin HRP solution at 37°C for 30 min. Finally, diaminobenzidine was used for color development and hematoxylin was used for counterstaining at 37°C for 15 min. The stained brain sections were observed using a Leica DM2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany), and images were captured using a digital camera and Leica software version 3.7 (Leica Microsystems GmbH). For quantification, five fields of view at x400 magnification were
randomly examined, and the number of brown cells in each field was counted by independent blinded operators. Brown spots were counted irrespective of whether they contained a blue nucleus. An immunohistochemical score (IHS) was calculated by multiplying the number of immunoreactive cells (quantity score) by the staining intensity (staining intensity score). Quantity scores were estimated as follows: No staining, 0; 1-10% of cells, 1; 11-50%, 2; 51-80%, 3; 81-100%, 4. Staining intensity was rated on a scale of 0-3 where: 0, negative; 1, weak; 2, moderate; and 3, strong. The IHS ranged from 0 to 12.

Western blotting. Proteins were extracted from the hippocampal and raphe nucleus tissue using cytoplasmic extracts (Beyotime Institute of Biotechnology, Jiangsu, China) and 10X PMSF (100:1). Protein concentration was detected using a bicinechonic acid working solution (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. A total of 30 µg protein/lane was separated via 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking of the membranes with 5% skim milk powder for 2 h at room temperature, they were incubated at 4°C overnight in primary antibodies against the following target proteins: SERT (cat. no. ab172884, 1:1,000, polyclonal rabbit; Abcam, Cambridge, UK), Bcl-2 (cat. no. AB112-1, 1:1,000, monoclonal rabbit; Beyotime Institute of Biotechnology), BDNF (1:1,000, polyclonal rabbit, cat. no. ab75040; Abcam, Cambridge, MA, USA), and β-actin (cat. no. EM32011-02, 1:1,000, monoclonal mouse; Beijing Emarbio Science and Technology, Beijing, China; www.emarbio.com). Subsequently, the membranes were washed and incubated in species-specific peroxidase-conjugated secondary antibodies for 2 h at room temperature. The secondary antibodies (all 1:6,000; HRP-conjugated) used to distinguish SERT, Bcl-2, BDNF (anti-rabbit, cat. no. A0208; Beyotime Institute of Biotechnology) and β-actin (anti-mouse, cat. no. A0216; Beyotime Institute of Biotechnology) were all produced in goats. The specific bands were detected using an Enhanced Chemiluminescence system (GE Healthcare, Chicago, IL, USA) and a Bio-Rad electrophoresis image analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RT-qPCR. Total RNA was extracted from hippocampal and raphe nucleus tissue samples using TRIzol® Reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions and quantified using a spectrophotometer (NanoDrop2000/2000C; Thermo Fisher Scientific, Inc.). Subsequently, the RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (cat. no. M1705; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and amplified using a Real-Time PCR Mx3000p Instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). RT-qPCR was performed using SYBR® Premix Ex Taq™ (with a pre-denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, primer annealing at 60°C for 30 sec; acquisition of the dissolve curve at 95°C for 15 sec; at 60°C for 30 sec; at 95°C for 15 sec) (cat. no. DRR041B; Takara Biotechnology Co., Ltd., Dalian, China). The primers for the target gene (forward primer, cat. no. SSD809230873; downstream primer, cat. no. SSD08926711; and reverse primer, cat. no. SSD809230181) and the reference gene U6 (forward primer, cat. no. SSD0904071006; downstream primer, cat. no. SSD0904071007; and reverse primer, cat. no. SSD0904071008) were designed and synthesized by Guangzhou RiboBio Co., Ltd (Guangzhou, China). After PCR, a melting curve was obtained to assess the quality of the reaction. The relative expression of miRNA was calculated as follows: 2^(-∆∆Cq) (ΔCt = Cq (TG) - Cq (RG); ∆∆Cq = ∆Cq (experimental) - ∆Cq (control)) (20).

Materials. Atropine, pilocarpine hydrochloride, paroxetine, trypsin, paraformaldehyde and the monoclonal antibody for BDNF were purchased from Sigma-Aldrich; Merck KGaA. The specific antibodies for Bcl-2, SERT and β-actin were purchased from Abcam. The TUNEL/HRP kit was purchased from Roche Applied Science (Penzberg, Germany). All other reagents were purchased from Biyuantian (Jiangsu, China).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism v6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance was performed with multiple comparisons between the groups using an Dunnett's post hoc tests and comparisons between the attack levels were performed using the Mann-Whitney test method. P<0.05 was considered to indicate a statistically significant difference.

Results

Behavioral alterations in chronic epileptic rats. At 3-10 min following the administration of pilocarpine, the animals exhibited masticatory movements, salivation, sniffing movements, tremors and partial seizures. At 15-30 min following pilocarpine injection, the animals developed SE that persisted for longer than 30 min. SE was successfully induced in all the epileptic group rats. The acute phase was followed by a quiescent phase of 2-7 days in which the animals behaved normally except for anorexia and hypokinesia. SSR-like activity was observed 8-27 days after the induction of SE. A total of 14 rats survived the induction of SE at 4 weeks; subsequently, 7 rats received paroxetine treatment and the other 7 rats were untreated. At the end of the experiment, 6 rats survived in each of the paroxetine-treated and untreated groups (total, n=12 rats). The frequency per week (P<0.0001; Fig. 1A) and the mean stage of the SSRs (P<0.05; Fig. 1B) were significantly decreased after paroxetine intervention compared with the SE group. The mortality of the rats may have been due to pilocarpine-induced epilepsy.

TUNEL/HRP staining in the hippocampus. Tissue sections of the hippocampus from the experimental groups were stained with TUNEL/HRP in order to evaluate apoptosis. With this assay, apoptotic neurons in the DG region were positively stained (brown) in the cytoplasm. In the vehicle group, positively stained neurons were sparse, and those that were positive only exhibited light positive staining (Fig. 2A). The number and IHS score of apoptotic neurons were increased in the SE group compared with the vehicle group (P<0.0001 and P<0.001, respectively; Fig. 2B and C). Following paroxetine
Figure 1. Behavioral alterations in chronic epileptic rats. (A) Frequency of SSRs per week in the chronic period. (B) Mean stage of SSRs in the chronic period. Data are presented as the mean ± standard deviation. *P<0.05 and ****P<0.0001 vs. SE. SSRs, spontaneous recurrent seizures; SE, status epilepticus.

Figure 2. Terminal deoxynucleotidyl transferase dUTP nick-end labelling/horse radish peroxidase staining in the hippocampus. (A) Representative staining images. Brown cells denote the apoptotic cells (examples pointed to by arrows; magnification, x400). (B) Quantification of the number of brown cells in 5 high power fields. (C) Quantification of the IHS scoring in the experimental groups. Data are presented as the mean ± standard deviation. ***P<0.001 and ****P<0.0001 vs. vehicle; #P<0.05 and ##P<0.01 vs. SE. IHS, immunohistochemical score; SE, status epilepticus; Pa, paroxetine.
intervention, the number of apoptotic neurons and IHS score significantly decreased compared with the SE group (P<0.001 and P<0.05, respectively; Fig. 2B and C).

**Expression of SERT, Bcl-2 and BDNF proteins.** SERT was expressed in both the raphe nucleus and the hippocampus in all experimental groups (Fig. 3A). In the hippocampus, SERT expression in the SE group was decreased compared with the vehicle group (P<0.01; Fig. 3A and B), but this effect was reversed by paroxetine, with SERT expression being significantly increased in the SE + paroxetine group compared with the SE group (P<0.05; Fig. 3A and B). In the raphe nucleus, SERT expression was decreased in the SE + paroxetine group compared with the SE group (P<0.05; Fig. 3A and B). The pattern of differences in Bcl-2 expression in the hippocampus was similar to that of SERT expression. Bcl-2 expression levels in the SE group were decreased compared with the vehicle group (P<0.0001; Fig. 3A and D), but following paroxetine intervention, Bcl-2 expression levels were significantly increased compared with the SE group (P<0.01; Fig. 3A and D). Additionally, in the hippocampus, BDNF expression levels in the SE group were decreased compared with the vehicle group (P<0.05; Fig. 3A and E); however, paroxetine intervention did not significantly alter BDNF expression compared with the SE group (Fig. 3A and D).

**Expression of miR-16.** In the hippocampus, miR-16 expression in the SE group was increased compared with the vehicle group (P<0.0001; Fig. 4A). Following paroxetine administration, miR-16 expression was significantly decreased compared with the SE group (P<0.05; Fig. 4A); however, miR-16 expression remained higher in the SE + paroxetine group than in the vehicle group (P<0.001; Fig. 4A). In the raphe nucleus, miR-16 expression in the SE group was decreased compared with the vehicle group (P<0.0001; Fig. 4B), and this increased expression was not significantly altered following paroxetine administration (Fig. 4B).
Discussion

Previous studies have demonstrated that serotonin serves an important role in epilepsy (21-23). In general, drugs that increase the level of extracellular serotonin, such as SSRIs/tryp- tophan and 5-hydroxytryptophan (5-HT) (21, 24, 25), alleviate seizures, whereas drugs such as 5,7-dihydroxytryptamine that reduce the level of serotonin may aggravate seizures (26, 27). However, the effects of SSRIs on seizures remain controver- sial. Previous clinical tests suggested that long-term treatment of depression with SSRIs increases the incidence of epilepsy. The rate of epileptogenesis in rats has been demonstrated to be enhanced by chronic SSRI treatment (28). However, clinical and animal experiments have demonstrated that SSRIs may decrease seizures, and these drugs are considered safe for use in epilepsy (3-5, 25). In the present study, four weeks of parox- etine treatment alleviated seizures in pilocarpine-induced chronic epileptic rats. Further studies will be required to determine the longer-term effects of SSRI treatment on epilepsy.

The molecular mechanism underlying the therapeutic role of SSRIs in epilepsy remains unclear. Our previous study revealed that in pilocarpine-induced epileptic rats, the level of extracellular serotonin in the hippocampus decreased, as did the number of 5-HTP-positive neurons in the raphe nucleus (6). SERT, which modulates 5-HT metabolism, is considered important for epilepsy, especially when it is accompanied by depression (29-32). Therefore, we hypothesized that SERT is abnormally expressed in pilocarpine-induced epileptic rats, although one study demonstrated the absence of a significant change in mRNA expression levels of SERT in this model (28). The present study demonstrated that SERT is expressed not only in the raphe nucleus but also in the hippocampus. No significant decreases in SERT expression in the raphe nucleus were observed in pilocarpine-induced chronic epileptic rats. Theoretically, it should decrease due to impairment of the raphe nucleus. It was hypothesized that the absence of an alteration in SERT expression reflects a form of self-regulation to ensure the availability of serotonin. Following paroxetine intervention, SERT was downregulated in the raphe nucleus, decreasing reuptake and thus increasing synaptic 5-HT avail- ability. Additionally, it was downregulated in the hippocampus in epileptic rats. This result is consistent with the results of Martinez et al (31), who demonstrated that SERT activity in the insula and fusiform gyrus was reduced in patients with temporal lobe epilepsy accompanied by depression. Following paroxetine intervention, SERT was upregulated in the hippocampus, indicating increased reuptake and therefore an increased level of serotonin in the hippocampus. Therefore, SERT expression alterations in pilocarpine-induced chronic epileptic rats differed across brain regions, and paroxetine treatment modulated the expression of SERT to increase the level of extracellular serotonin in the hippocampus.

The question remains as to why SERT expression levels are altered in epilepsy. Previous studies have focused on the epigenetic and genetic pathogenesis of epilepsy (33-36), under the assumption that one gene modulates a number of proteins and one protein may be regulated by various different genes. miRNA is a one example, as selective alterations in miRNAs that regulate neuronal cell death, ion channels and inflammation have been identified in epileptic patients and in experimental epileptogenic models (8, 10, 11, 13, 37, 38). In a genome wide miRNA profiling study, miR-16 expression was increased in the hippocampus of patients with MTLE (9). Similarly, the present study demonstrated that miR-16 was upregulated in the hippocampus in pilocarpine-induced chronic epileptic rats. However, following paroxetine intervention, it was downregulated. By contrast, in the raphe nucleus, miR-16 was downregulated, demonstrating that the alteration in miR-16 expression in chronic epileptic rats had brain tissue specificity. The pattern of change in miR-16 expression was opposite to that of SERT. In addition, miR-16 has been reported to target SERT, and in experimental models of depression, paroxetine may upregulate miR-16 expression in the raphe nucleus (7). Therefore, it may be hypothesized that miR-16 may have a role in regulating the gene expression of SERT in the raphe nucleus and hippocampus of chronic epileptic rats.

Another question that remains is whether other proteins are targeted by miR-16. Recent experimental results have suggested that miR-16 may regulate the cell cycle and apoptosis in tumors (16, 39, 40), including T lymphoblastic lymphoma/leukemia, breast cancer, glioma and hepatocel- lular carcinoma. For example, Mobarra et al (41) revealed that miR-16 overexpression reduces Cyclin D1 and Bcl-2 expression and increases apoptosis in breast cancer cells. Recent studies have demonstrated that miR-16 may target BDNF (42, 43). In general, miR-16 overexpression may downregulate BDNF and therefore inhibit cell proliferation, including in depression models and SH-SY5Y cells (42, 43). Temporal lobe epilepsy
is characterized by hippocampal sclerosis, including neuronal apoptosis and glial proliferation. As an antiapoptotic protein, Bcl-2 has been demonstrated to regulate mitochondrial permeability and caspase-3 activity in epilepsy (44,45), whereas the association between BDNF and seizures remains controversial. One study revealed that upregulating BDNF may increase epilepsy susceptibility (46), and another reported that upregulating BDNF alleviates seizures in pilocarpine-induced epileptic mice (47). This suggested that continuously injecting an appropriate amount of BDNF into the hippocampus may alleviate kainic acid-induced seizures via the promotion of neuronal regeneration and therefore demonstrated that BDNF serves a protective role in neuronal apoptosis (47). In the present study, obvious neuronal apoptosis and downregulation of Bcl-2 and BDNF expression were observed in pilocarpine-induced chronic epileptic rats. Paroxetine alleviated neuronal apoptosis and upregulated Bcl-2 expression. In the present study, Bcl-2 exhibited an opposite trend of expression than miR-16, and therefore it may be hypothesized that miR-16 overexpression downregulated Bcl-2 expression and increased neuronal apoptosis in chronic epileptic rats. However, the association between BDNF and miR-16 in epileptic rats remains uncertain.

The mechanism by which miR-16 targets SERT, Bcl-2 and BDNF requires further study. In addition, one protein may be regulated by a number of miRNAs. For example, SERT is regulated by miR-16, in addition to miR-55 and other miRNAs (48,49). Therefore, further studies are required to determine whether SERT, Bcl-2 and BDNF are primarily targeted by miR-16.

In conclusion, the present study demonstrated that seizures and hippocampal apoptosis in chronic epileptic rats may be alleviated by paroxetine treatment, which may be associated with alterations in SERT and Bcl-2/BDNF protein expression. The alterations in miR-16 expression may provide a potential explanation for the modulation of apoptosis. Further study is required to determine the underlying molecular mechanisms.

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